Immunity, Vol. 8, 233–243, February, 1998, Copyright ©1998 by Cell Press

Altered Antigen Presentation in Mice Lacking H2-O

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Summary

HLA-DM catalyzes the release of MHC class II–associated invariant chain–derived peptides (CLIP) from class II molecules. Recent evidence has suggested that HLA-DO is a negative regulator of HLA-DM in B cells, but the physiological function of HLA-DO remains unclear. Analysis of antigen presentation by B cells from mice lacking H2-O (the mouse equivalent of HLA-DO), together with biochemical analysis using purified HLA-DO and HLA-DM molecules, suggests that HLA-DO/H2-O influences the peptide loading of class II molecules by limiting the pH range in which HLA-DM is active. This effect may serve to decrease the presentation of antigens internalized by fluid-phase endocytosis, thus concentrating the B cell–mediated antigen presentation to antigens internalized by membrane immunoglobulin.

Introduction

Immediately after synthesis in the endoplasmic reticulum (ER), major histocompatibility complex (MHC) class II molecules associate with the invariant chain (Ii). Ii inhibits the binding of peptides and nascent proteins to class II molecules in the ER and directs Ii–class II complexes to the endosomal system, where binding of antigenic peptides occurs (Wolf and Ploegh, 1995). Ii is degraded by proteolysis, but complete removal of class II–associated invariant chain peptides (CLIP) requires the catalytic function of HLA-DM (DM), a resident of the endosomal/lysosomal system that is structurally related to class II molecules (Fling et al., 1994; Karlsson et al., 1994; Morris et al., 1994; Sanderson et al., 1994). The absence of DM (or H2-M, the equivalent mouse molecule) leads to the accumulation of CLIP-containing class II molecules and decreased loading of antigenic peptides (Mellins et al., 1990; Fung-Leung et al., 1996; Martin et al., 1996; Miyazaki et al., 1996). In vitro, DM is sufficient to release CLIP peptides from class II molecules, but this effect is not restricted to CLIP (Sloan et al., 1995; Weber et al., 1996; Kropshofer et al., 1997). Thus, the dissociation rate of any peptide from class II molecules appears to be increased in the presence of DM but remains proportional to the intrinsic dissociation rate of the peptide (Weber et al., 1996; Kropshofer et al., 1997). Whether DM-mediated release of peptides other than CLIP is also important in vivo is presently unknown.

In B cells, the majority of DM is associated with HLA-DO (DO) (Liljedahl et al., 1996), and a recent report has shown that association with DO inhibits the ability of DM to release CLIP, both in vitro and in transfected cells (Denzin et al., 1997). The physiological relevance of this effect is unclear, however, since CLIP-containing class II molecules are not particularly prominent on B cells—the main, if not exclusive, cell type expressing DO (Tonelle et al., 1985; Wake and Flavell, 1985; Karlsson et al., 1991; Liljedahl et al., 1996; Douek and Altmann, 1997).

In contrast to dendritic cells, which are comitted to antigen presentation irrespective of antigen specificity (Cella et al., 1997), B cells are themselves antigen specific, but usually require T cell help in order to mature into antibody-secreting plasma cells (Vitetta et al., 1991). The antigens presented by B cells are internalized mainly by their membrane immunoglobulin receptors (mIg) (Rock et al., 1984; Lanzavecchia, 1985); receptor-independent antigen presentation by B cells, though well studied in vitro, is relatively inefficient. How B cells focus antigen presentation to antigens internalized by the B cell receptor is unclear, since increased antigen capture can only partly explain this phenomenon (Watts, 1997). It has been suggested that efficient B cell receptor–mediated antigen presentation may require specialized loading compartments (Mitchell et al., 1995; Watts, 1997), and several groups have described class II–rich intracellular compartments (Peters et al., 1991; Amigorena et al., 1994; Tulp et al., 1994; West et al., 1994). These compartments are not restricted to B cells, however (Calafat et al., 1994; Kleijmeer et al., 1994), and their functional importance is unclear. Although the general machinery for antigen processing is likely to be the same in different antigen-presenting cells, it is still possible that cell type–specific differences in antigen processing may contribute to the efficient presentation of antigens internalized by mIg.

We generated mice lacking H2-Oa to evaluate the importance of H2-O/DO for peptide loading and antigen presentation. Cells from H2-Oa–deficient mice were found to have normal levels of class II expression, and the density of H2-A^b–CLIP complexes at the cell surface was the same as in wild-type controls. However, B cells from H2-Oa–deficient mice were found to have changed capacity to present protein antigens when compared to B cells from wild-type mice, suggesting that the absence of H2-O does modify the peptide content of class II molecules at the cell surface, either qualitatively or quantitatively. In older H2-Oa–deficient mice, serum levels of immunoglobulin G1 (IgG1) were elevated, further

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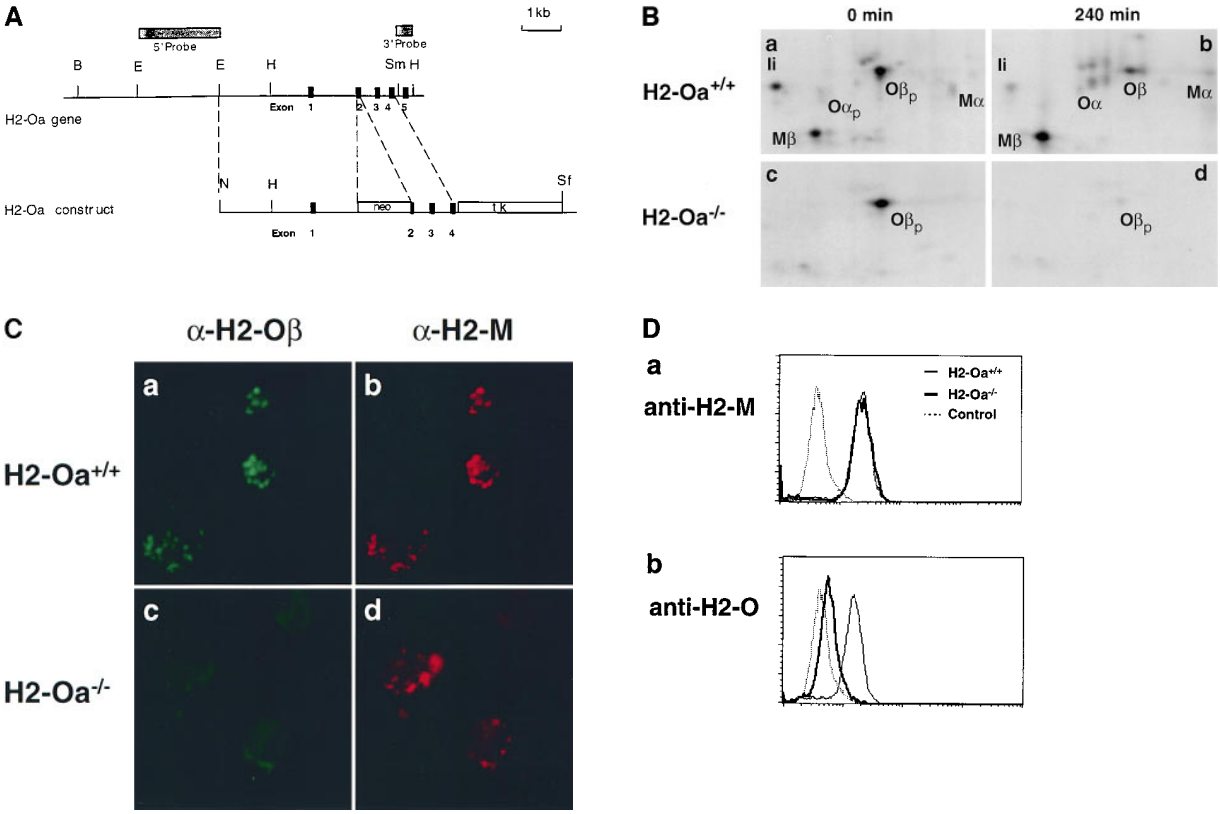

Figure 1. Disruption of the Mouse H2-Oa Gene

(A) The mouse H2-Oa gene and the knockout construct are shown. A neomycin-resistance gene (*neo*) was inserted into exon 2 of the H2-Oa gene, and a herpes simplex–thymidine kinase gene (*tk*) was placed at the 3′ end of the construct. Restriction sites are BamHI (B), EcoRI (E), HindIII (H), NotI (N), SfiI (Sf), and SmaI (Sm). Numbered filled boxes are exons. The probes shown flanking the 5′ and 3′ ends of the construct were used in Southern hybridization to confirm homologous recombinations.

(B) Immunoprecipitation from ^{35}S-labeled spleen cells. H2-Oa$^{+/+}$ (top) or H2-Oa$^{-/-}$ (bottom) splenocytes were labeled for 30 min (a and c) and either analyzed immediately (0 min) or after chase in nonradioactive medium for 240 min (b and d). H2-Oβ was immunoprecipitated from the cell lysates with rabbit antiserum K535 (anti-H2-Oβ) and analyzed by two-dimensional gel electrophoresis. Oαp and Oβp indicate nontransported forms of Oα and Oβ. Acidic proteins are located to the right.

(C) Confocal images of H2-Oa$^{+/+}$ (a and b) or H2-Oa$^{-/-}$ (c and d) splenocytes stained with K535 (a and c) and 2E5A (anti-H2-M) (b and d).

(D) Analysis of H2-O and H2-M expression by flow cytometry. B cells from H2-Oa$^{+/+}$ (fine line) or H2-Oa$^{-/-}$ (bold line) mice were permeabilized with saponin and stained with 2E5A for H2-M (a) or K535 for H2-Oβ (b). The negative control staining (dotted line) was the same in the two types of mice.

suggesting that T cell–B cell interaction may be changed in these mice. Analysis of DO function in vitro, using recombinant molecules, confirmed the finding by Denzin et al. (1997) that DO inhibits DM function, but showed that the inhibition is decreased at an acidic pH, suggesting that peptide loading may be favored in acidic compartments (probably lysosomes) in the presence of DO.

Together, our data suggest that DO/H2-O is a pH-dependent inhibitor of DM/H2-M, and that the main influence of DO/H2-O expression in vivo may not be to decrease CLIP release but rather to modify the peptide-editing function of DM, probably by restricting the location where DM is fully active. This action may serve to focus antigen presentation to antigens internalized by mIg.

Results

Generation and Characterization of H2-Oa–Deficient Mice

Embryonic stem cells lacking a functional H2-Oa gene were generated by homologous recombination, as outlined in Figure 1A. The disruption of the H2-Oa gene was confirmed by Southern blotting. The modified embryonic stem cells were used to derive homozygous mice in which both of the H2-Oa alleles were disrupted.

To confirm the absence of H2-Oα protein, splenocytes from wild-type mice (H2-Oa$^{+/+}$) or from H2-Oa–deficient mice (H2-Oa$^{-/-}$) were metabolically labeled for 30 min, and H2-O was immunoprecipitated using an H2-Oβ antiserum, either directly or after a 4 hr chase in nonradioactive medium. In the absence of H2-Oα, the H2-Oβ chain

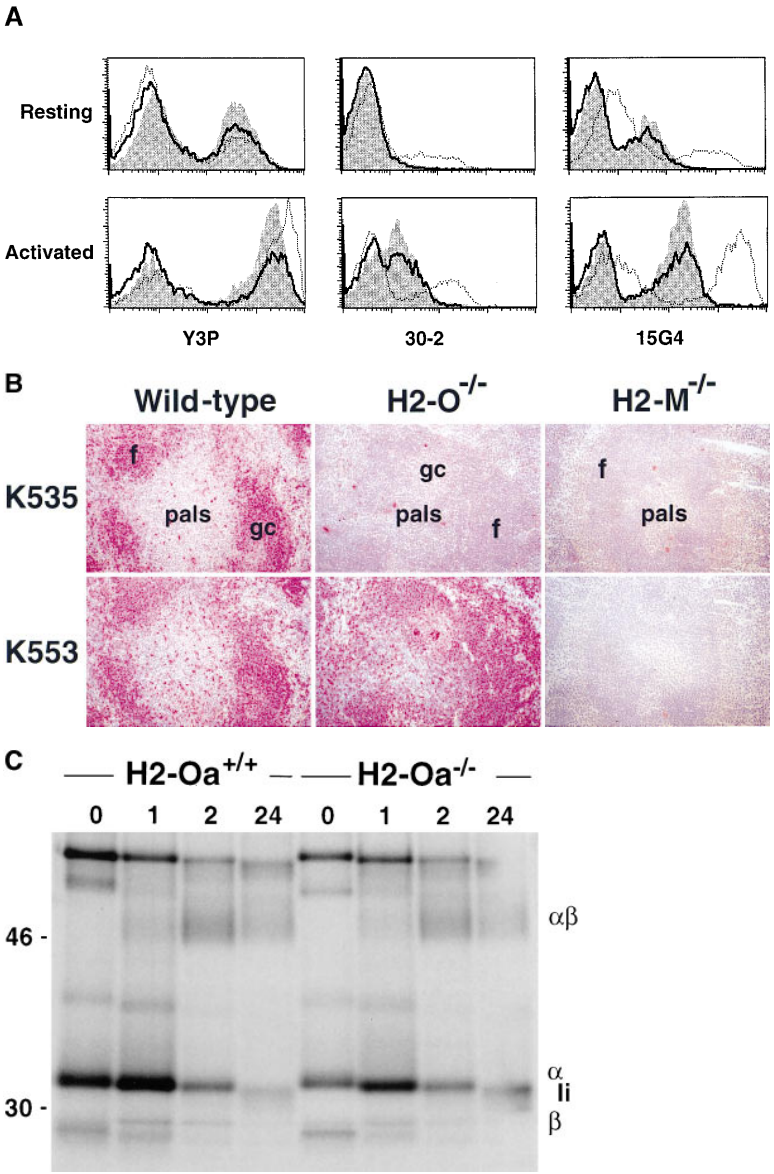

Figure 2. Characterization of MHC Class II Expression in H2-Oa$^{-/-}$ Mice and H2-O$^{+/+}$ Littermates

(A) Lymph node cells from wild-type (shaded area), H2-Oa$^{-/-}$ (solid line), and H2-M$^{-/-}$ (dotted line) mice were stained with antibodies reactive to H2-A^b (Y3P) or CLIP-containing H2-A^b (30-2 and 15G4) and analyzed by flow cytometry.

(B) Serial spleen sections from wild-type, H2-Oa$^{-/-}$, and H2-M$^{-/-}$ mice were stained for H2-O or H2-M with rabbit antiserum K535 or K553, respectively. Locations of follicles (f), periarteriolar lymphocyte sheath (pals), and germinal centers (gc) are depicted.

(C) Immunoprecipitation from ^{35}S-labeled spleen cells. H2-Oa$^{+/+}$ (left) or H2-Oa$^{-/-}$ (right) splenocytes were labeled for 30 min and then either analyzed immediately (0 min) or after chase in nonradioactive medium for the indicated time (in hours). H2-A^b was immunoprecipitated and samples were analyzed without prior boiling or reduction. Size markers (at left) are in kilodaltons.

had a decreased half-life and did not associate with H2-M or acquire any carbohydrate modifications, indicating that it was not transported out of the ER (Figure 1B). Indirect immunofluorescence staining of H2-Oa$^{+/+}$ splenocytes with the H2-Oβ antiserum showed the expected vesicular staining pattern (Liljedahl et al., 1996) (Figure 1Ca), whereas the staining in H2-Oa$^{-/-}$ B cells was barely detectable (Figure 1Cc). Staining with an H2-M–reactive monoclonal antibody (mAb) showed that this molecule had the same distribution whether or not H2-O was present (compare Figures 1Cb and 1Cd). Analysis of permeabilized B cells by flow cytometry showed similar levels of H2-M in wild-type and H2-Oa$^{-/-}$ B cells (Figure 1Da). In contrast, H2-Oβ staining was barely detectable in the mutant cells (Figure 1Db).

Class II Expression in H2-Oa–Deficient Mice

In mice lacking H2-M, the cell surface levels of H2-A^b are normal, but the peptide content of these molecules is changed, so that the vast majority of molecules contain CLIP. The class II expression at the cell surface of lymph node cells from H2-Oa–deficient mice was analyzed by flow cytometry. As controls, cells from wild-type and H2-M–deficient mice were analyzed. The density of H2-A^b expression on H2-Oa$^{-/-}$ lymph node cells (which contain both B and T cells) was found to be similar to the density on wild-type cells, whether the cells were resting or had been activated by treatment with lipopolysaccharide (LPS) and interleukin-4 (IL-4). Thus, mAbs that bind to H2-A^b irrespective of the bound peptides, such as Y3P (Figure 2A) and M5/114, as well as mAbs that bind to H2-A^b in a peptide-specific manner, such as BP107 and KH74 (data not shown), stained H2-Oa$^{-/-}$ and H2-Oa$^{+/+}$ cells equally well. In contrast H2-M–deficient mice were weakly stained by KH74 and not at all by BP107, as has been reported previously (Fung-Leung et al., 1996) (data not shown). The staining with 30–2, a mAb which specifically recognizes CLIP-H2-A^b complexes was very

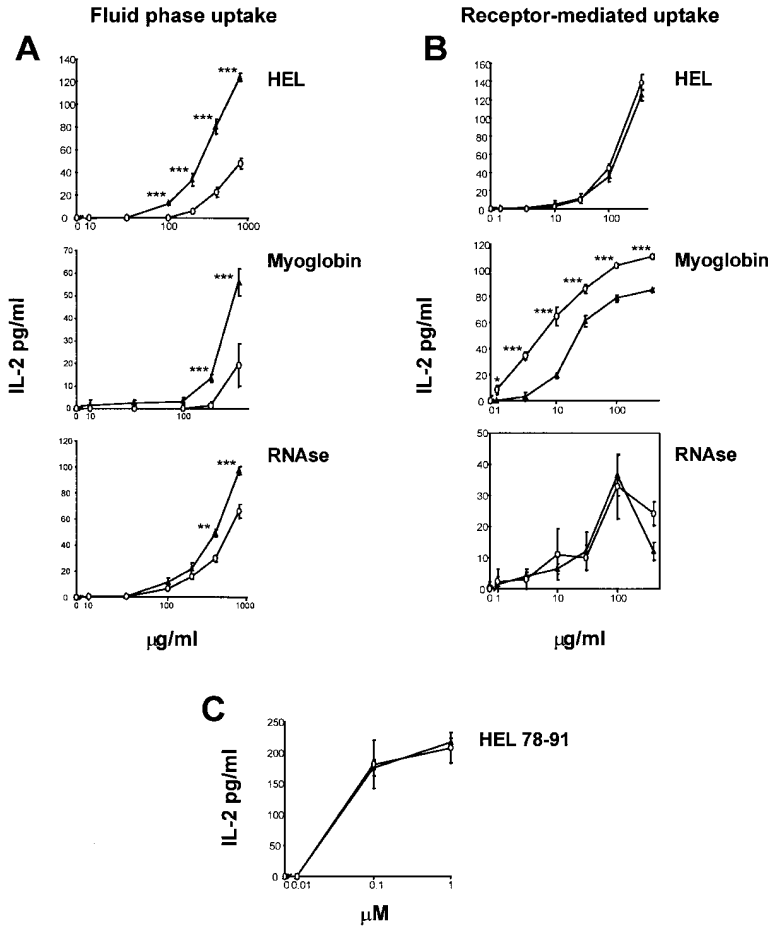

Figure 3. Antigen Presentation by B Cells and Serum Immunoglobulin Levels

(A) Presentation of antigens internalized by fluid phase to T cell hybridoma cells. Highly purified B cells from H2–$O^{+/+}$ (circles) and H2-$O^{-/-}$ (triangles) mice were incubated with 0–800 μg/ml antigen (up to 400 μg/ml myoglobin) and T hybridoma cells overnight. IL-2 production by the hybridoma cells was measured in culture supernatants by ELISA.

(B) Presentation of antigens internalized by mIg-mediated uptake. B cells from transgenic H2–$O^{+/+}$ (circles) and H2-$O^{-/-}$ (triangles) mice expressing anti-pc antibody were pulsed with pc-conjugated antigens for 1 hr, washed extensively to remove the excess antigen, and incubated with hybridoma cells overnight. Values represent the mean (± SD) IL-2 production from triplicate cultures.

Levels of statistical significance between the means using Student's t test are indicated (*$P < 0.05$; ** $P < 0.01$; *** $P < 0.005$).

(C) Peptide presentation to HEL hybridoma cells. B cells from H2-$O^{+/+}$ (circles) and H2-$O^{-/-}$ (triangles) mice were cultured overnight (18 hr) with 0–1 μM HEL peptide (amino acids 74–91) in the presence of the HEL hybridoma. IL-2 production was measured as described above.

weak on resting wild-type and H2-$O^{-/-}$ B cells, while H2-$M^{-/-}$ B cells were stained well with this antibody. Activation by treatment with LPS and IL-4 increased the 30–2 staining on B cells from all three types of mice, but there was still no difference in staining intensity between wild-type and H2-$Oa^{-/-}$ mice. MAb 15G4 also recognizes CLIP-H2-A^b complexes but with higher affinity than 30–2 (P. Wong and S. Y. Rudensky, manuscript in preparation). The staining with this antibody also showed comparable CLIP-H2-A^b levels on lymph node cells from wild-type and H2-$Oa^{-/-}$ mice, whether the cells were resting or had been activated, while the staining on cells from H2-$M^{-/-}$ mice was very intense. The staining increased on cells from all three types of mice after activation (Figure 2A).

Immunohistochemical analysis of the spleen and lymph node from H2-$Oa^{-/-}$ mice with an antiserum against H2-Oβ confirmed the absence of H2-O expression, which in wild type mice is limited mostly to B cells (Figure 2B) (Wake and Flavell, 1985; Karlsson et al., 1991). As expected from biochemical data (Liljedahl et al., 1996), H2-O was undetectable in the spleen and lymph node of H2-$M^{-/-}$ mice, while H2-M expression was normal in H2-$Oa^{-/-}$ mice (Figure 2B). Unexpectedly, however, the H2-Oβ–specific antiserum gave staining patterns identical to wild-type mice (Karlsson et al., 1991; Surh et al., 1992) on thymic sections from both H2-$Oa^{-/-}$ and H2-$M^{-/-}$ mice; strong staining was observed on medullary epithelial cells with weaker staining on cortical epithelial cells (Surh et al., 1992; C. D. S., unpublished data). The stainings were specific, since binding of the antibodies was blocked in the presence of the H2-Oβ peptide used to raise the antiserum. We are presently investigating this finding.

Under mildly denaturing conditions, H2-A^b molecules from both wild-type and H2-M–deficient mice migrate as dimers on SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Fung-Leung et al., 1996; Martin et al., 1996; Miyazaki et al., 1996). The migration of the dimers is not identical, however, and the finding that H2-A^b molecules from H2-M–deficient mice are loaded almost exclusively with CLIP peptide explains this phenomenon. Analysis of H2-A^b molecules immunoprecipitated from metabolically labeled H2-$Oa^{+/+}$ or H2-$Oa^{-/-}$ splenocytes using mAb M5/114 showed that these molecules migrated identically on SDS-PAGE, whether derived from the wild-type or from the mutant cells, suggesting that the class II molecules in H2-$Oa^{-/-}$ mice contain a mixture of peptides, like the class II molecules from wild-type mice (Figure 2C).

Immune Functions in the Absence of H2-O

The proportions of B cells as well as of $CD4^+$ and $CD8^+$ T cells in the lymph node, spleen, and thymus were

similar in H2-Oa–deficient and wild-type mice (data not shown). Lymph node $CD4^+$ T cells, which displayed a naive phenotype when analyzed by CD44 and CD45RB, reacted normally against allogenic stimulator cells. Likewise, we have been unable to find significant differences in the ability of spleen cells (depleted of T cells) from mutant or wild-type mice to stimulate alloresponses (data not shown). This finding is not unexpected, since dendritic cells, which express little or no H2-O, are thought to be the main cell type mediating the response in this assay (Sprent, 1995). B cells had normal surface expression of IgM, IgD, CD5, CD16, CD19, CD22, CD23, CD45R, and HSA (data not shown).

To determine the capacity of B cells from H2-$Oa^{-/-}$ mice to process and present protein antigens, B cells from wild-type and mutant mice were used to stimulate IL-2 production from a panel of T cell hybridomas reactive with different antigens. Figure 3A shows that although all the antigens could be presented by the wild-type cells to some extent after overnight incubation with the antigen, hen egg lysozyme (HEL) and sperm whale myoglobin were distinctly better presented by the H2-$Oa^{-/-}$ B cells, while the difference for RNAse was smaller (but seen consistently in several experiments). To evaluate whether antigens internalized by mIg also were differentially presented, H2-$Oa^{-/-}$ or wild-type litter-mates were bred with 207-4 mice, carrying transgenes for phosphorylcholine (pc)–specific immunoglobulin μ and κ chains. B cells from these mice were pulsed for 60 min with pc-conjugated antigens and analyzed for their ability to stimulate the same panel of hybridomas as mentioned above. In this situation, the presentation of myoglobin by wild-type cells was superior to the presentation by the H2-$Oa^{-/-}$–deficient cells, while HEL and RNAse were presented equally well by the two types of presenting cells (Figure 3B). There was no difference in the presentation between nonconjugated and pc-conjugated antigens when tested after fluid-phase uptake (data not shown). B cells from mutant or wild-type mice were equally efficient at presenting HEL peptide 74-91 to the HEL-reactive hybridoma (Figure 3C).

The increased presentation of antigens internalized by fluid-phase endocytosis could potentially lead to less stringent requirements for delivery of T cell help and thus for the expansion of B cells. An indication that this may be the case was obtained when the serum levels of immunoglobulins were analyzed in nonimmunized (nontransgenic) mice. While young H2-Oa–deficient and wild-type animals (6 weeks of age) had similar immunoglobulin levels of all subclasses (data not shown), older H2-Oa–deficient mice (10 months of age) had distinctly increased levels of IgG1 compared to the wild-type controls (Figure 4). Other IgG subclasses and IgA did not show significant differences between the two types of mice.

Analysis of DM-DO Interaction In Vitro

The similar levels of CLIP-H2-A^b complexes in both wild-type and mutant mice suggested that H2-O does not effectively inhibit CLIP release in vivo, yet the changed presentation of antigenic epitopes showed that H2-O did influence peptide presentation, either qualitatively or quantitatively. To investigate the molecular basis for this phenomenon, recombinant soluble DO, DM, and DR molecules as well as DMDO complexes were produced in insect cells (Jackson et al., 1992; Matsumura et al., 1992). Human molecules rather than mouse molecules were chosen, since DM function in vitro has been better characterized in the human system (Denzin and Cresswell, 1995; Sloan et al., 1995; Weber et al., 1996; Kropshofer et al., 1997). Both transmembrane and truncated soluble forms of DO are unstable in the absence of DM, and only minor amounts of DO exit the ER (Liljedahl et al., 1996 and Figures 1B and 1C) To overcome this problem, we fused the extracellular domains of DOA and DOB to the Fc domain of human IgG1, thus creating a DO–Fc fusion protein (below called DO). DMDO complexes were isolated from cells expressing all four DM and DO chains. Soluble DM and DR molecules were generated as described in Experimental Procedures. The activity of the recombinant DM and DMDO molecules was tested for their ability to promote peptide loading of a fluorescent peptide from hemagglutinin (HA) (amino acids 306–318) to DR1. At pH 5.5 the activity of the recombinant molecules was found to be very similar to what has been described for affinity-purified molecules from B cells (Denzin et al., 1997), confirming that DO has an inhibitory effect on DM function (Figure 5A).

DM can release peptides other than CLIP from class II molecules (Weber et al., 1996; Kropshofer et al., 1997), and the changed antigen presentation in the H2-$Oa^{-/-}$ mice could be a reflection of changed specificity for peptide release of the H2-M–H2-O complexes compared with H2-M alone. We analyzed the release of several different peptides from DR1 in vitro (shown for CLIP and HA in Figures 5B and 5C, respectively) but were not able to find any differences in specificity between DMDO and DM. In contrast, at pH 5.5, DMDO complexes consistently appeared to release peptides with slower kinetics than DM alone.

DO Is a pH-Dependent Inhibitor of DM Function

Alterations in pH often result in changed protein conformation and changed protein function. To investigate whether structural changes occurred in the purified proteins during acidification, we used the fluorescent probe ANS (1-anilino-naphtalene-8-sulphonic acid). ANS fluorescence is low in aqueous solutions but strongly increased when the probe becomes associated with exposed hydrophobic protein surfaces (Stryer, 1968; Boniface et al., 1996; Runnels et al., 1996). While the ANS fluorescence in samples containing DM alone did not increase significantly upon acidification (Figure 6A), the fluorescence in DO and DMDO-containing samples increased drastically, suggesting a change in DO conformation at lower pH. However, although DO may contribute most of the conformation change in the DMDO complexes, it cannot be excluded that the conformation of the associated DM molecules is also altered. The conformation change in DO-containing samples was independent of the DO Fc domain since the fluorescence of CD27Fc did not increase at acidic pH. Incubation of DM or DMDO complexes at the indicated pH for 4 hr did

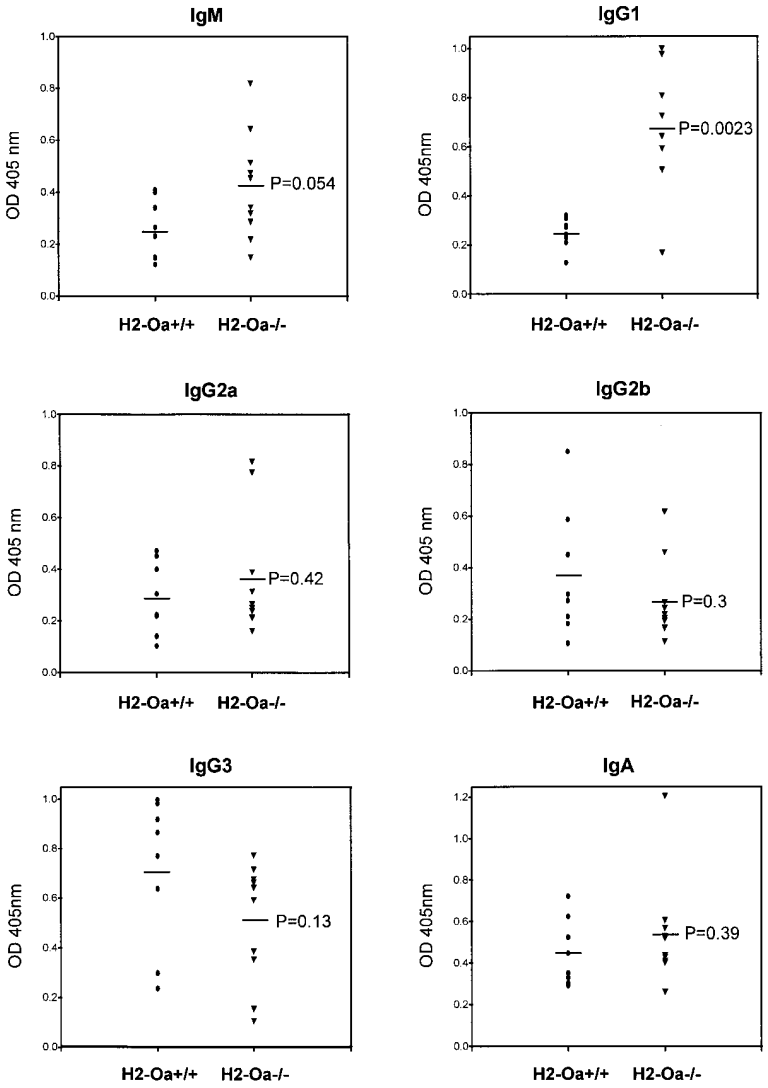

Figure 4. Immunoglobulin Levels in Sera

Sera from 10-month-old sex-matched H2-O$^{+/+}$ (circles) and H2-O$^{-/-}$ (triangles) mice were titrated and immunoglobulin levels were measured using isotype-specific ELISA. Bars represent mean serum levels expressed as optical density (OD) at 405 nm.

not further increase the ANS fluorescence (Figure 6B). Neutralization of the samples containing DO restored the original level of fluorescence, suggesting that the conformational changes were reversible.

DM is efficient in the pH range between 6 and 4.5 (although the optimal pH for peptide loading to class II molecules is peptide and haplotype dependent) (Jensen, 1991; Denzin and Cresswell, 1995; Sherman et al., 1995; Sloan et al., 1995). To determine whether the conformational change in DO correlated with the capacity for peptide exchange of DMDO complexes, peptide binding to DR1 was analyzed at different pH levels in the presence of equimolar amounts of DM or DMDO. While DM was found to catalyze loading of HA peptide to DR1 throughout the expected pH range (in this case with optimal loading at pH 5.5), DMDO complexes were inactive at higher pH but promoted peptide loading almost as well as DM at pH 4.5 (Figure 7A). A similarly increased activity of the DMDO complex at pH 4.5 compared to pH 5.5 was seen for the loading of HLA-A2 peptide to DR1 and (to a smaller extent) for the association of a peptide from a mycobacterial heat shock protein (HSP 65) to DR3 (data not shown).

To exclude the possibility that the Fc domain of DO was influencing the function of the DMDO complexes, this domain was removed by papain digestion and the capacity of the modified complex to catalyze peptide loading was analyzed. Figure 7B shows that the papain-digested complexes, as well as DMDO complexes formed in vitro from free DM and DO promoted peptide loading with kinetics similar to those of the in vivo–formed DMDO complexes, both at pH 5.5 and at pH 4.5.

At equilibrium, a certain amount of free DM is present in the DMDO complex preparation (since the interaction is noncovalent). To ascertain that the pool of free DM present at neutral pH was not sufficient to mediate the peptide loading we detected with the DMDO complexes (if for example the DMDO complexes were irreversibly destroyed), binding experiments were done at pH 5.5 and pH 4.5 using DMDO complexes and two concentrations of DM. Comparison of the loading kinetics showed that while the higher concentration of DM (64 nM) was almost as effective as DMDO at pH 5.5 (Figure 7C), DMDO was distinctly better at the lower pH (Figure 7D), suggesting either a release of more free active DM from the DMDO complexes upon acidification or a change in

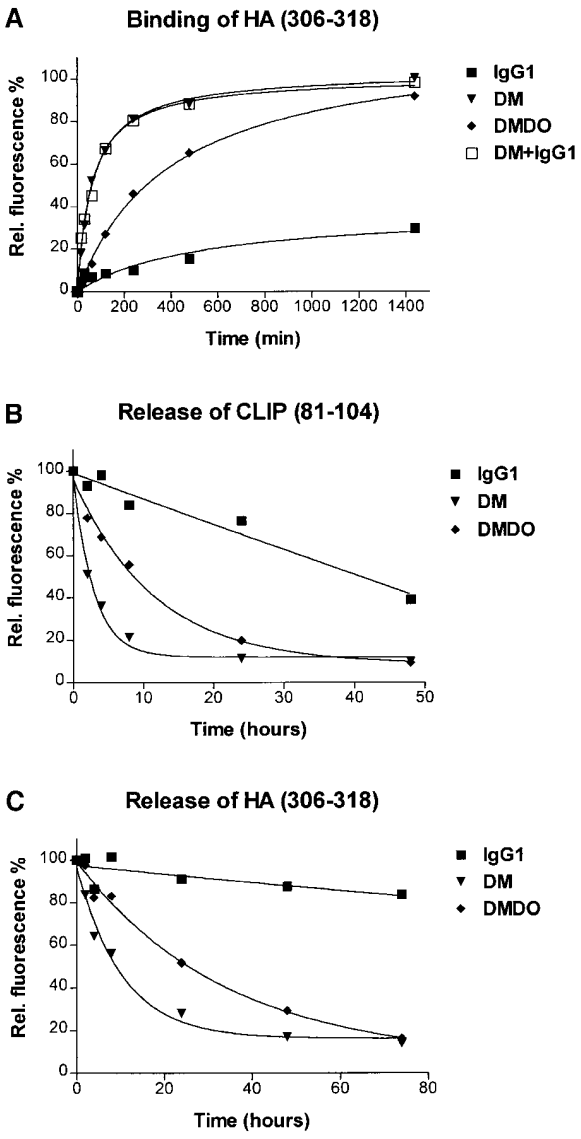

Figure 5. Analysis of DO Function In Vitro

(A) Kinetics of FITC-HA binding to DR1. DR1 (500 nM) was incubated with FITC-HA peptide (2.5 μM) alone or with the indicated proteins (DM 500 nM, DMDO 500 nM, or DM 500 nM and IgG1 500 nM) for increasing lengths of time at 37°C, pH 5.5. Samples were neutralized and free peptide was separated from DR–peptide complexes by gel filtration. A fluorescence of 100% represents the binding in the presence of DM.

(B and C) Kinetics of FITC-CLIP (B) and FITC-HA (C) release from DR1. DR1 preloaded with FITC-labeled peptides was incubated with 5 μM unlabeled HA peptide alone or in the presence of DM (500 nM) or DMDO (500 nM). Samples were treated as described above. Fluorescence is given as the percentage of the initial value.

the DMDO complex that increased catalytic function at the lower pH.

Discussion

In this study we used H2-Oa–deficient mice to address the functional relevance of H2-O for antigen processing and presentation. We found that H2-O influences the

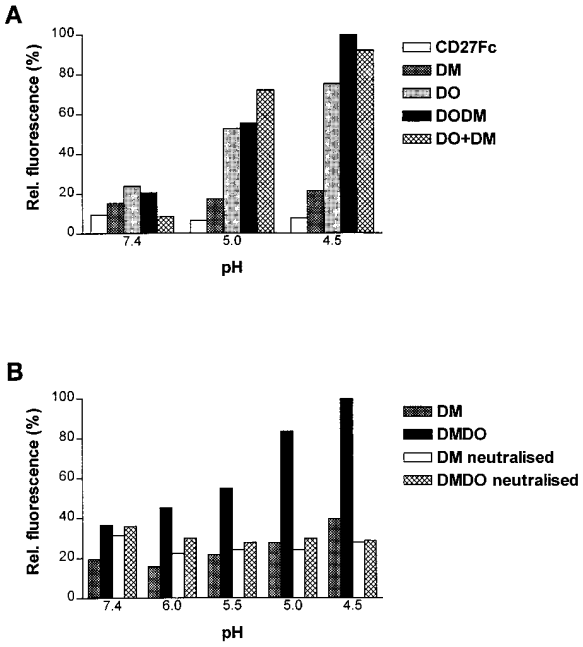

Figure 6. Conformational Changes in DO and DMDO upon Acidification

(A) Proteins (at 200 nM) were incubated at the indicated pH. ANS (to 20 μM) was added and fluorescence was measured immediately.

(B) DM or DMDO (at 200 nM) were incubated at the indicated pH for 4 hours at 37°C. ANS (to 20 μM) was added and fluorescence was measured immediately. Samples were neutralized and remeasured immediately.

loading of peptides to class II molecules (i.e., H2-A^b) in B cells, since protein antigens were presented differently to antigen-specific T cell hybridomas depending on whether or not the presenting B cells expressed H2-Oa. When the analyzed antigens were internalized by fluid-phase endocytosis, all were presented better (to varying degrees) by B cells from H2-Oa–deficient mice than by B cells from wild-type mice. In contrast, the same antigens were either presented better or equally well by B cells from wild-type mice when internalized by a transgenic mIg, suggesting that the presence of H2-O results in discrimination between different forms of antigen uptake.

The H2-A^b in H2-Oa–deficient mice appear to contain a mixture of peptides and have essentially normal reactivity with anti-H2-A^b antibodies. Furthermore, the cell surface expression of CLIP-H2-A^b was found to be essentially identical on both resting and activated B cells from wild-type or H2-Oa–deficient mice. This finding was somewhat unexpected, considering that Denzin et al. (1997) have convincingly shown that expression of DO inhibits DM-mediated CLIP release in transfected cell lines, resulting in increased surface levels of CLIP–class II complexes.

It is not obvious why expression of an additional molecule inhibiting DM function would be more favorable than controlling the transcription of DM itself, unless DO either changes the specificity of DM or provides a mechanism for more rapid shifts in DM activity than can be achieved by transcriptional regulation. Cellular

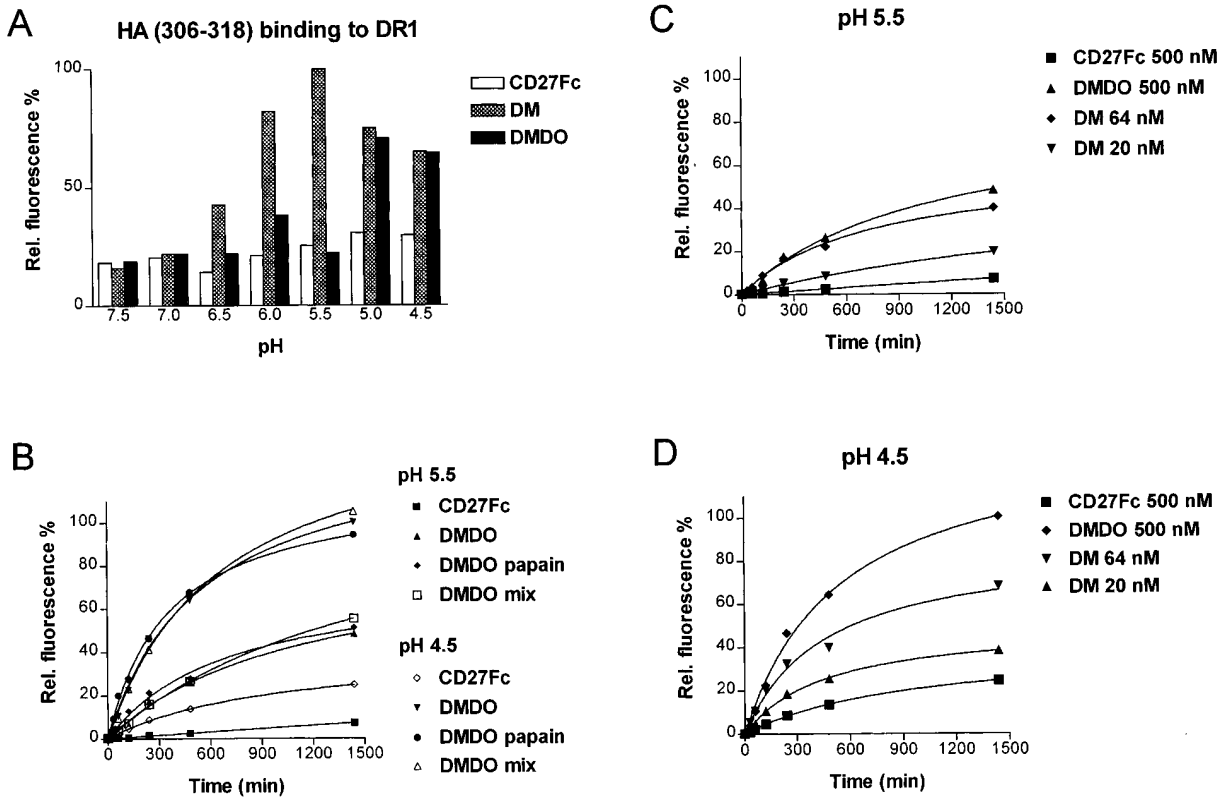

Figure 7. Increased Activity of DMDO at Acidic pH

(A) CD27Fc, DM, or DMDO (125 nM) was incubated at 37°C for 3 hr at the indicated pH before addition of DR1 (250 nM) and FITC-HA peptide (5 μM). Samples were incubated for 1.5 hr at 37°C and then neutralized and analyzed as in Figure 5. A fluorescence of 100% represents the binding in the presence of DM at pH 5.5.

(B) Kinetics of FITC-HA binding to DR1 (500 nM) in the presence of CD27, DMDO, papain-digested DMDO, or in vitro–formed DMDO complexes (DMDO mix), all at 500 nM. Samples were incubated for increasing lengths of time at 37°C at pH 5.5 or pH 4.5 and then analyzed as in Figure 5. A fluorescence of 100% indicates the maximal fluorescence in the presence of DMDO at pH 4.5.

(C and D) Kinetics of FITC-HA binding to DR1 (500 nM) in the presence of CD27 (500 nM), DMDO (500 nM), and two dilutions of DM (64 nM and 20 nM) at pH 5.5 (C) or pH 4.5 (D). Samples were incubated at and analyzed as described above. A fluorescence of 100% indicates the maximal fluorescence in the presence of DMDO at pH 4.5.

activation or signal transduction events could potentially result in the dissociation of DMDO complexes, thus releasing free DM. However, cross-linking of mIg on B cell lines or splenocytes, as well as antigen-nonspecific stimulation with LPS or phorbol esters, do not result in readily detectable dissociation or posttranslational modifications of DMDO (or H2-M–H2-O) complexes (M. L. and L. K., unpublished data). Although we cannot totally exclude such events, since transient or subtle changes would be difficult to detect using standard biochemical techniques, there is presently no evidence to suggest that DM activity would be controlled by intracellular signaling events.

The specificity of peptide loading could be changed either directly or indirectly by the presence of DO/H2-O. A possible direct effect was analyzed using purified recombinant molecules, and in this system we were able to confirm the previously published data (Denzin et al., 1997) (obtained with detergent-solubilized material from B cell lines) showing that DMDO complexes were distinctly less active than DM alone in mediating CLIP release. However, peptides other than CLIP also appeared to be released from class II molecules with slower kinetics by DMDO than by DM, suggesting that DO did not directly change the specificity of DM-mediated peptide release.

An alternative explanation, supported by our biochemical data, is that DO indirectly influences the specificity of DM-mediated peptide release by limiting the pH interval in which DM is fully active. Thus, while DM was active in a relatively broad pH range, between pH 6.0 and 4.5, the DMDO complexes had low activity at pH greater than 5.5. At lower pH, however, the DMDO complexes were almost as active as DM alone, suggesting that association with DO decreases the pH optimum for DM-mediated peptide exchange, or rather that association with DO narrows the pH range where DM is active. It is not clear whether DMDO complexes dissociate at acidic pH to release free active DM, or whether the complexes interact directly with DR molecules. We have not been able to detect DR–DMDO complexes, but presently it cannot be excluded that they exist. It should be pointed out that some free DM undoubtedly also exists in the endocytic pathway if DO is expressed, since the two molecules are noncovalently associated.

The pH-dependent differences in activity between DM

and DMDO complexes suggest that the differences in antigen presentation observed between the H2-Oa–deficient and the wild-type mice may reflect a skewing of the localization for peptide loading to more acidic compartments in the presence of H2-O. This could potentially result either in decreased or increased presentation of a certain epitope, depending on where it becomes accessible as well as how sensitive it is to destruction. The competition by other peptides for binding to class II molecules will also influence the probability of presentation of particular epitopes. In addition, different class II molecules have varying pH requirements for peptide loading, and this factor is also likely to influence how they are affected by the presence or absence of H2-O. Preferred peptide loading in lysosomes could be advantageous in the case of B cells, in which relevant antigens are internalized by mIg (Rock et al., 1984; Lanzavecchia, 1985). Protein domains are often stabilized by their interaction with antibodies (Accolla et al., 1981; Jemmerson and Paterson, 1986; Simitsek et al., 1995), and high-affinity antigen–antibody complexes may require lysosomal conditions for the release of antigenic epitopes, due to the protease resistance of the antibody itself. Decreased H2-M activity in endosomal compartments (due to the association with H2-O) would limit the risk of presenting antigens internalized by fluid-phase or low-affinity receptors, without seriously limiting the presentation of antigens internalized by high-affinity mIg. The elevated IgG1 titers in the H2-O–deficient mice may reflect increased activation of B cells in response to antigens taken up by fluid-phase or low-affinity receptors. During an immune response, activation of $CD4^+$ T cells by B cells presenting peptides from such antigens could potentially result in the expansion of T and B cells with irrelevant or harmful specificities. This could result in the dilution of an effective immune response, but also in the expansion of autoreactive cells, thus increasing the risk for autoimmune reactions. The presence of H2-O/DO in B cells may thus serve to focus antigen presentation to antigens internalized by mIg in order to increase the specificity of the immune response and to avoid reactivity to self antigens.

Experimental Procedures

Gene Targeting

A 4.3 kb DNA fragment from a 129/Ola mouse genomic clone covering most of the H2-Oa gene was used in the knockout construct. A cassette containing a neomycin-resistance gene was inserted into exon 2. A herpes simplex–thymidine kinase cassette was placed at the 3′ end of the construct. The DNA construct was introduced into day 14 embryonic stem cells by electroporation. Cells were cultured in the presence of 400 μg/ml G418 and 0.2 μM ganciclovir. Embryonic stem cells with the targeted gene were detected by polymerase chain reaction and then confirmed by Southern hybridization using DNA probes flanking the construct.

$H2\text{-}Oa^{-/-}$ or $H2\text{-}Oa^{+/+}$ mice were bred with transgenic 207-4 mice (Storb et al., 1986) (on a C57BL/6 background). Expression of the transgene was tested with enzyme-linked immunosorbent assay (ELISA) against pc-conjugated protein.

Metabolic Labeling and Immunoprecipitation

Splenocytes were labeled with [^{35}S]methionine and [^{35}S]cysteine as indicated before lysis in 1% Triton X-100, phosphate-buffered saline (PBS), and Complete proteinase inhibitor cocktail (Boehringer Mannheim, Germany). H2-O was immunoprecipitated with a rabbit antiserum (K535) against the H2-Oβ cytoplasmic tail (Karlsson et al., 1991). $H2\text{-}A^b$ was precipitated with M5/114 (Bhattacharya et al., 1981). Immunoprecipitates were harvested with protein A- or G-Sepharose, washed, and resuspended in isoelectric focusing sample buffer (Figure 1B) (Jones, 1980) or SDS-PAGE sample buffer containing 2% SDS without reduction. Samples were left at room temperature for 20 min and then separated on 7.5%–12.5% polyacrylamide gels directly or after nonequilibrium pH-gradient electrophoresis (pH 3.5–10). Gels were fixed, dried, and autoradiographed. Autoradiographs were scanned using an Agfa Arcus II scanner. Composites were printed on a Kodak XLS 8600 printer.

Immunofluorescence

Cells were attached to coverslips coated with Cell-Tak (Collaborative Biomedical Research, Bedford, MA) before fixation with 4% formaldehyde–PBS. After fixation, cells were washed with 50 mM NH_4Cl, PBS. Antibody incubations were made in PBS with 0.6% fish skin gelatin and 0.2% saponin for permeabilization. Fluorescein isothiocyanate (FITC)–labeled anti-rabbit immunoglobulin (Cappel) and Texas Red–labeled anti-mouse or anti-rat immunoglobulin (Molecular Probes) secondary reagents were used. Fluorescent cells were imaged using a Bio-Rad confocal microscope.

Flow Cytometry

Lymph node and spleen cells were stained with biotinylated anti-IgM (μ-specific) (Jackson ImmunoResearch), M5/114, Y3-P, 30–2, BP107, KH74, B220, or J11d followed by FITC-streptavidin (Jackson ImmunoResearch). For 15G4 (mouse IgG1), anti-IgG (Fcγ-specific $F(ab')_2$) was used for secondary staining. Cells were also double-stained with biotinylated anti-CD5, CD16, CD19, CD22, CD23, or IgD followed by FITC-streptavidin and phycoerythrin (PE)–conjugated anti-B220. Cells from lymph node and thymi were double-stained with FITC-conjugated anti-CD8 and PE-labeled anti-CD4 as previously described (Surh et al., 1992). For staining of permeabilized cells, B cells were stained in 1% fetal calf serum with 0.1% azide and 0.02% saponin without fixation using biotinylated K535 (anti-H2-Oβ) or 2E5A (anti-H2-M) (Fung-Leung et al., 1996) followed by FITC-streptavidin.

Immunohistochemistry

Cryostat sections were stained for H2-O or H2-M with rabbit antisera against the H2-Oβ tail (K535) or against H2-M (K553) (Karlsson et al., 1994), respectively, followed by biotinylated anti-rabbit IgG (Jackson ImmunoResearch). Bound antibodies were detected with alkaline phosphatase–conjugated streptavidin (Jackson ImmunoResearch) and visualized with colorimetric substrate as described (Surh et al., 1992).

Antigen Presentation Assays

B cells were purified from pooled lymph node and spleen cells by passage over G-10 columns and complement-mediated depletion of T cells by antibodies directed against CD4 (RL 172) and CD8 (3.168), followed by a 90 min incubation on plastic tissue culture dishes to remove any remaining adherent cells (Webb and Sprent, 1990). Purified B cells (1×10^5) were incubated in triplicate overnight with 2×10^5 hybridoma cells recognizing sperm whale myoglobin (HMb 4.2.2, kindly provided by P. Jensen, Emory University, Atlanta, GA), HEL (BO4), and ribonuclease A (IB-E6) using increasing amounts of relevant antigen or HEL peptide (amino acids 74–91). Recombinant sperm whale myoglobin (Sigma), HEL, and ribonuclease A (Calbiochem) were purchased. Cultured hybridoma supernatants were tested for the presence of IL-2 using ELISA according to the manufacturer's instructions (Genzyme).

Receptor-mediated uptake and presentation to the panel of hybridomas was studied after a 1 hr pulse with pc-conjugated antigens (Gearhart et al., 1975), to B cells purified from 207-4 anti-pc transgenic $H2\text{–}O^{+/+}$ or $H2\text{-}O^{-/-}$ mice, followed by extensive washing.

Data are shown as mean values ± SD when indicated. Student's t test was used to determine levels of significance between sample means using Sigma plot 3.0 (Jandel).

None of the antigens is presented by antigen-presenting cells from $H2\text{-}M^{-/-}$ mice (Martin et al., 1996; O. W., unpublished data).

Peptides

Peptides were synthesized using F-moc chemistry. A fluorescein-labeled lysine precursor was incorporated during synthesis of HA (306–318, sequence PKYVKQNTLKLAT). CLIP (81–104, sequence LPKPPKPVSKMRMATPLLMQALPM) was labeled at the amino terminus with FITC (Molecular Probes).

Typing of Antisera

Isotype-specific ELISAs were performed on sera from sex- and age-matched 6-week-old or 10-month-old mice according to the manufacturer's instructions (Southern Biotechnology Associates).

Recombinant Proteins

cDNAs were modified by PCR. The transmembrane domain was deleted in DRA and DRB1; the transmembrane and cytoplasmic tails of DMA and DMB were replaced by six histidines followed by a stop codon. DO was expressed as a fusion protein in which the transmembrane and cytoplasmic tails of DOA and DOB were replaced by human IgG1 Fc domains. CD27Fc has been described (Ozaki et al., 1997). Constructs were cloned into expression vector pRMHa-3 and expressed in *Drosophila melanogaster*–derived Schneider-2 cells (Matsumura et al., 1992). Soluble proteins were purified from the culture medium by affinity chromatography followed by gel filtration. For the initial purification step a mAb LB3.1 column was used for DR1 as described (Stern and Wiley, 1992); nickel nitrilotriacetic acid agarose (Ni-NTA agarose; Qiagen) columns were used for DM and DMDO complexes; and protein A-Sepharose columns were used for DO and CD27Fc. Superdex 200 columns (Pharmacia) were used for gel filtration. DMDO complexes were digested with immobilized papain (Pierce) according to the manufacturer's instructions. Fc domains were removed using protein A-Sepharose. DMDO complexes were also formed in vitro by incubating DM with 4-fold excess DO for 4 hr at room temperature. Complexes were isolated using Ni-NTA agarose beads. These complexes behaved identically to DMDO complexes isolated from cells expressing both DM and DO. The correct identities of all proteins were confirmed by amino-terminal protein sequencing. DMDO complexes contained equimolar amounts of all four chains. Protein concentrations were determined using bicinchoninic acid (BCA; Pierce). Human IgG1 was purchased from Sigma.

Peptide Exchange Assays

DR–peptide complexes were formed by incubation of 100 times molar excess of peptide with recombinant DR for 48 hr at pH 6.0. DR–peptide complexes were separated from free peptide by gel filtration using a Superdex 75 column (Pharmacia).

Binding reactions were done using DR1 (preloaded with nonfluorescent CLIP 81–104, except as stated) with 5- to 20-fold excess of peptide at 37°C in 45 mM HEPES, 2-(N-morpholino) ethanesulfonic acid (MES), or sodium acetate buffer (depending on pH) with 100 mM NaCl. Reactions were stopped by addition of Tris-HCl pH 8.0 to 600 mM and frozen until analysis. DR-peptide complexes were separated from free peptide by gel filtration over Sephadex G-50 (Pharmacia) columns (2 ml bed volume). Bound fluorescence was measured using either a Shimadzu single sample fluorometer or a Perseptive Cytofluor 96 well fluorometer. Samples were excited at 485 nM and emission was analysed at 530 nM.

ANS Fluorescence

200 nM of the indicated proteins were incubated in buffer (20 mM HEPES, MES, or sodium acetate). ANS was added to a final concentration of 20 μM either immediately or after 4 hr of incubation. After ANS addition, samples were excited at 372 nm and emission was analyzed at 480 nm. Samples were neutralized by the addition of HEPES (pH 7.4) to 100 mM as indicated. Emission spectra (410–600 nm) were also analyzed, but the wavelength of fluorescence maximum did not substantially shift upon acidification.

Acknowledgments

We thank D. Uranowski and K. Sedat for secretarial assistance; J. Kenny for 207-4 mice; P. Jensen for T cell hybridomas; S. Baker, L. Hatlen, P. Ling, V. Moreno, and J. Pang for technical assistance; the members of the DNA core laboratory and peptide laboratories for DNA sequencing and for oligonucleotide and peptide synthesis; and M. Jackson, J. Sprent, R. Teasdale, and S. Webb for discussions and comments.

Received December 3, 1997.

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